Original article

Anionic antimicrobial peptide-lysozyme interactions in innate pulmonary immunity

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Abstract

The respiratory tract contains numerous antimicrobial factors necessary for normal innate pulmonary defense. Although many of these molecules reside in airway surface liquid (ASL) simultaneously, little information exists concerning antagonistic, additive, or synergistic interactions. Since both cationic lysozyme and anionic antimicrobial peptides (AP) are found in high concentrations in ASL, the purpose of this study was to assess any interaction that might affect antimicrobial activity. For this, *Pasteurella haemolytica*, *Micrococcus lysodeikticus*, or *Pseudomonas aeruginosa* were added to egg white lysozyme (3.9–250.0 μg/ml) or human neutrophil lysozyme (0.8–50.0 μg/ml) and H-GADDDDD-OH (from 0.01 to 0.50 mM) mixtures in 50, 100, or 150 mM NaCl; incubated for 2 h; and then plated. In this assay, the MICs of AP for *P. haemolytica*, *M. lysodeikticus*, and *P. aeruginosa* varied slightly depending upon the concentration of NaCl and MICs generally increased slightly with increasing NaCl concentrations. The MIC of lysozyme for *P. haemolytica* and *M. lysodeikticus* also increased slightly with increasing NaCl concentrations. The MIC of lysozyme for *P. aeruginosa* was greater than 50 μg/ml and did not vary with increasing NaCl concentrations. When AP was combined with lysozyme in 50, 100, or 150 mM NaCl concentrations, there was no significant interaction that affected antimicrobial activity. In conclusion, the MICs of AP generally increased with increasing NaCl concentrations but lysozyme and AP appeared not to interact significantly at physiologically relevant concentrations. © 1999 Elsevier Science B.V. and International Society of Chemotherapy. All rights reserved.

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1. Introduction

The respiratory tract contains numerous antimicrobial factors necessary for normal innate pulmonary defense. Some of these factors are undefined [1,2]. Others include anionic peptides (AP) [3,4], β-defensins [5–7], α-defensins [8], antimicrobial surfactant proteins [9], lysozyme [10], secretory leukoprotease inhibitor [11], lactoferrin [12], human salivary mucin glycoprotein MG2 [13], human salivary histatin 5 [14], and cathelicidin [15]. Despite differences in concentration, molecular mass, mechanism of action, and ionic charge, many of these molecules are thought to reside in airway surface liquid (ASL) simultaneously. However, little information exists on the antagonistic, additive, or synergistic interactions affecting antimicrobial activity.

AP and lysozyme predominate in ASL and reach concentrations of 0.1–1.3 mg/ml [4] and 1–10 μg/ml, respectively. AP, originally isolated from ovine pulmonary surfactant [3] consists of three small (721.6–823.7 Da), hydrophilic peptides containing homopolymeric regions (e.g., 5–7 residues) of aspartic acid. AP are thought to be produced in respiratory epithelium [4]. MICs for AP and similar analogues are comparable to those of other vertebrate antimicrobial peptides [16]. Lysozyme, one of the best-characterized hydrolytic enzymes, is large, cationic and produced by respiratory epithelium and alveolar macrophages. Lysozyme hydrolyses the polysaccharide portion of

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bacterial cell walls [17,18]. However, this enzyme may have other important physiological functions [19].

Although AP and lysozyme predominate in ASL, they are opposite in a number of characteristics including molecular mass, mechanisms of antimicrobial activity, and ionic charge which may be antagonistic to effective antimicrobial activity. However, since they have different mechanisms of antimicrobial activity, they may also be synergistic (e.g. lysozyme opening the cell wall allowing a smaller AP molecule to penetrate). Therefore, in this study, we were interested to see if AP and lysozyme concentration dependent interactions occurred and were influenced by the ionic microenvironment using *Pasteurella haemolytica*, *Micrococcus lysodeikticus*, or *Pseudomonas aeruginosa*.

2. Materials and methods

2.1. Bacterial strains and cultivation

Pasteurella haemolytica serotype A1 strain 82-25, *Micrococcus lysodeikticus*, and *Pseudomonas aeruginosa* O1 were used. *Pasteurella haemolytica* strain 82-25 and *M. lysodeikticus* were grown in tryptose broth at 37°C with constant stirring for 3 h. *Pseudomonas aeruginosa* O1 was grown in LB broth at 37°C with constant stirring for 16 h. After incubation, cultures were centrifuged at $5900 \times g$ for 5 min at 4°C. The bacterial pellet was resuspended in 100 mM NaCl, adjusted in the spectrophotometer (78% transmittance, 600 nm; Coleman model 35; Bacharach Instrument Co.) to contain 1.0×10^8 cfu/ml, and diluted 10^{-4} fold in 100 mM NaCl to contain 10^4 cfu/ml.

2.2. Peptide synthesis

H-GADDDDD-OH (721.7 Da) was synthesized by Multiple Peptide Systems (San Diego, CA) as described previously [4]. Peptide was 95–99% pure and verified by amino acid analysis. For the antimicrobial assay, H-GADDDDD-OH was suspended in distilled water, adjusted to a $4 \times$ stock solution of 2.0 mM and diluted two-fold to 0.03 mM.

2.3. Lysozyme

Egg white lysozyme (Worthington Biochemical Corporation, Lakewood, New Jersey), used in assays with *P. haemolytica* and *M. lysodeikticus*, was suspended in 100 mM NaCl, adjusted to a $4 \times$ stock solution of 1000 µg/ml 100 mM NaCl, and diluted two-fold to 15.64 µg/ml for *P. haemolytica* and 0.24 µg/ml for *M. lysodeikticus*. Human neutrophil lysozyme (Calbiochem-Novabiochem Corporation, La Jolla, CA), used in the assay with *P. aeruginosa*, was suspended in

100 mM NaCl, adjusted to a $4 \times$ stock solution of 200 µg/ml 100 mM NaCl, and diluted to 3.12 µg/ml.

2.4. Susceptibility test

A dilution susceptibility test was used to obtain a minimal inhibitory concentration (MIC) [16]. Since AP and lysozyme are sensitive to divalent cation concentration [20], salt concentration [2,21], pH, and buffer composition [1,3,22], the following modifications were necessary to assess antimicrobial activity. Dilutions of the $4 \times$ stock solution of H-GADDDDD-OH (50 µl, 2–0.03mM) were put in decreasing order into the wells of rows A–G of styrene plates (Immulon 1, Dynex Technologies, Inc., Chantilly, VA USA) and 50 µl of distilled water was put into the wells of row H. Dilutions of the $4 \times$ stock solutions of lysozyme (50 µl) were put in decreasing order into the wells of column 1–11 and 50 µl of 100 mM NaCl was put into the wells of column 12. To adjust the final concentration of NaCl to 50, 100, or 150 mM, 50 µl of distilled water, 200 mM NaCl, or 400 mM NaCl were added, respectively, to all wells. Finally, bacterial culture (50 µl), diluted as described above, was added to each dilution and the plates were incubated at 37°C for 120 min. Control wells contained only 50, 100, or 150 mM NaCl both with and without H-GADDDDD-OH and lysozyme. After incubation, the number of viable bacteria was determined by culturing 150 µl from each well in triplicate (50 µl/per spot) on trypticase soy agar containing 5% defibrinated sheep blood and incubating the plates overnight at 37°C. MIC values of AP (mM) in dilutions of lysozyme were determined as the highest dilution of peptide showing no growth (e.g., $\geq 90\%$ killing of controls) on blood agar.

3. Results

In this assay, the MICs of AP for *P. haemolytica*, *M. lysodeikticus*, and *P. aeruginosa* varied depending upon the concentration of NaCl in the incubation mixture. MICs generally increased with increasing NaCl concentrations (Table 1).

The MIC of lysozyme for *P. haemolytica* and *M. lysodeikticus* also increased with increasing NaCl concentrations (Table 1). The MIC of lysozyme for *P. aeruginosa* was greater than 50 µg/ml and did not vary with increasing NaCl concentrations.

When AP was combined with lysozyme in 50, 100, or 150 mM NaCl solutions, there was no significant interaction that affected antimicrobial activity (Fig. 1). In high egg white lysozyme concentrations (> 63 µg/ml), the MIC of AP for *P. haemolytica* was only slightly increased (Fig. 1A). In low egg white lysozyme concentrations (< 63 µg/ml), there was no effect and the only

differences seen in AP MIC were due to the effect of NaCl concentration.

In high concentrations of egg white lysozyme (e.g., greater than 0.97 µg/ml), no AP MIC could be detected for *M. lysodeikticus* due to the lytic action of egg white lysozyme at this concentration (Fig. 1B). However, when the concentration of egg white lysozyme fell below 0.48 µg/ml, the MIC of AP was consistently 0.12 mM regardless of NaCl concentration.

The concentration of human neutrophil lysozyme did not affect the MIC of AP for *P. aeruginosa* (Fig. 1C). However, the MIC of AP was consistently lower in 50 mM NaCl than in 100 mM and 150 mm NaCl.

4. Discussion

The presence of many molecules simultaneously in ASL suggests that interactions may occur that may be syngeristic or antagonistic. However, little information exists on any of these relationships. Since AP and lysozyme are present in concentrations needed for in vitro antimicrobial activity, we were interested to see if any interaction occurred that had an effect on antimicrobial activity. In this study, lysozyme did not affect the MIC of AP for *P. haemolytica*, *M. lysodeikticus*, and *P. aeruginosa*. This is in contrast with other studies that show synergistic interactions among antimicrobial substances and lysozyme. For example, Schnapp and Harris [8] showed that HD-1–3 acted synergistically with each other and with lysozyme. Similarly, Bals et al. [15] showed that the MIC for LL-37/hCAP-18 was decreased in the presence of lactoferrin and lysozyme, also suggesting synergism between peptides and

Table 1
Susceptibility of *Pasteurella haemolytica*, *Micrococcus lysodeikticus*, and *Pseudomonas aeruginosa* to anionic peptide and lysozyme

Organism	Saline (mM)	AP MIC (mM)[a]	Lysozyme MIC (µg/ml)
P. haemolytica[b]	50	0.030	ND
	100	0.030	125
	150	0.060	>250
M. lysodeikticus[c]	50	0.060	1
	100	0.125	2
	150	0.125	2
P. aeruginosa[d]	50	0.250	>50
	100	0.500	>50
	150	0.500	>50

[a] Anionic peptide (AP), H-GADDDDD-OH.
[b] *Pasteurella haemolytica* serotype A1, strain 82-25.
[c] *Micrococcus lysodeikticus*.
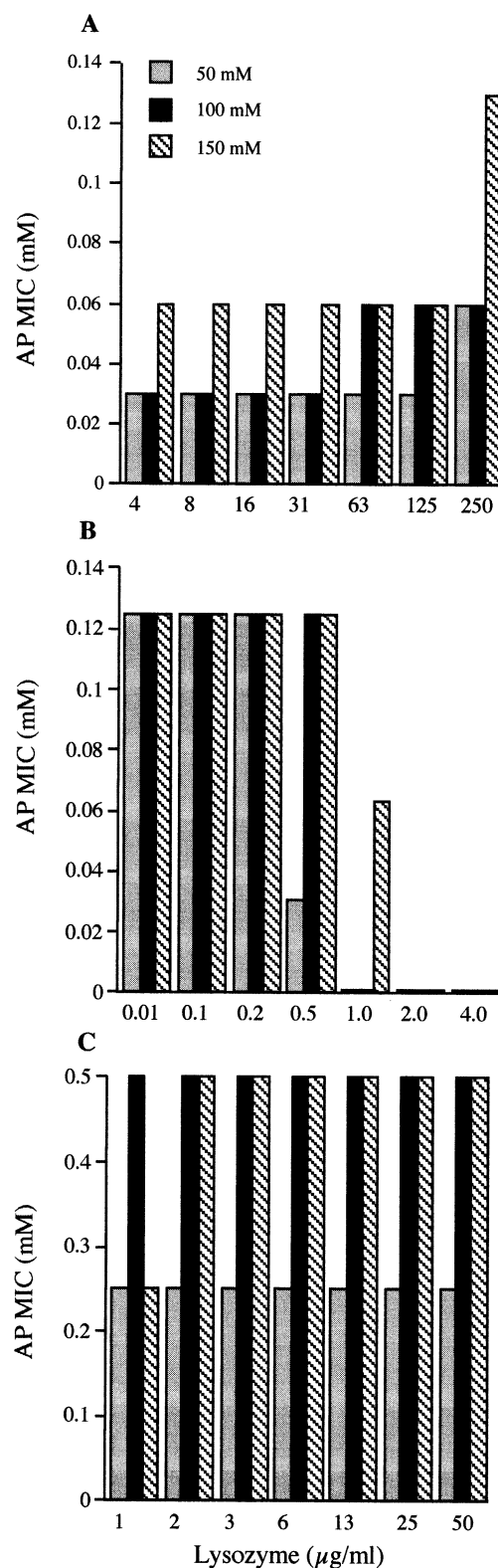[d] *Pseudomonas aeruginosa* strain PAO1.

Fig. 1. The effect of sodium chloride on the MIC of the anionic peptide, H-GADDDDD-OH, for: (A) *Pasteurella haemolytica* serotype A1 strain 82-25; (B) *Micrococcus lysodeikticus;* and (C) *Pseudomonas aeruginosa* PAO1 at varying concentrations of egg white lysozyme (graphs A and B) and human neutrophil lysozyme (graph C).

proteins in ASL. Selsted et al. showed that lysozyme enhanced the killing activity of rat defensins against *P. aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli*. Synergism was observed at a defensin concentration of 50 µg/ml but not at 25 µg/ml, suggesting that the defensins may primarily exert some effect on disintegration of the outer membrane of Gram-negative bacteria [23,24]. The lysozyme was thought to attack the peptidoglycan and the defensins contributing to the microbicidal activity by increasing the cell membrane permeability and several other functions [23–25].

Our results suggest that lysozyme and AP, despite differences in molecular mass, mechanisms of antimicrobial activity, and ionic charge, do not significantly interact in either an antagonistic, additive, or synergistic fashion. Although lysozyme effectively lysed *M. lysodeikticus* in these assay conditions, the concentrations of lysozyme needed for a similar effect on *P. haemolytica* or *P. aeruginosa* were artificially higher than lysozymes concentrations normally seen in ASL as well as the effective concentrations needed for lysis in other assays. Nevertheless, even under these conditions, lysozyme did not significantly affect the MIC of AP.

Many antimicrobial peptides are inactivated in NaCl concentrations greater than 100 mM. For example, Bals et al. [15] demonstrated that bacterial killing of LL-37 was sensitive to NaCl. Antimicrobial activity was diminished 4–5 fold over a range of NaCl in ASL that distinguishes patients without cystic fibrosis (CF) from patients with CF (e.g., 80 mM in fluid from patients without CF vs. 140 mM NaCl in fluid from patients with CF) [15,26,27]. It has been suggested that inactivation of hBD-1 due to elevated salt concentration in the lungs of patients with CF contributes to their susceptibility to chronic lung infection. Our results also suggest that the concentration of NaCl can influence the MIC of AP.

In conclusion, The MICs of lysozyme and AP generally increased with increasing NaCl concentrations but lysozyme and AP appeared not to significantly interact at physiologically relevant concentrations to increase antimicrobial activity.

Acknowledgements

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